Selected Ion Monitoring Method for Determination of Nicotine, Cotinine and Deuterium-labeled Analogs: Absence of an Isotope Effect in the Clearance of (S)-Nicotine-3',3'-d$_2$ in Humans

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A method for simultaneous determination of nicotine, its metabolite cotinine, and the stable isotope-labeled analogs nicotine-3',3'-d$_2$ and cotinine-4',4'-d$_2$ in human plasma has been developed. The method utilizes capillary column gas chromatography with detection by electron impact mass spectrometry and selected ion monitoring. Sensitivity is adequate for determination of nicotine and nicotine-d$_2$ at concentrations as low as 1 ng ml^{-1}, and cotinine and cotinine-d$_2$ at concentrations as low as 10 ng ml^{-1} with good precision and accuracy. The method has been used to compare the elimination kinetics of (S)-nicotine-3',3'-d$_2$ with natural nicotine in human subjects. Total clearance of nicotine-3',3'-d$_2$ was virtually identical to the total clearance of natural nicotine, which validates the use of the deuterium-labeled analog in quantitative studies of nicotine metabolic disposition.

INTRODUCTION

Stable isotope methodology is frequently used to study the metabolic disposition of drugs under conditions of chronic dosing and to determine bioavailability.[1] By administering a drug labeled with a stable isotope and measuring concentrations mass spectrometrically, quantitative aspects of metabolism can be studied without altering the usual dosing regimen. This is especially useful for drugs which may induce or inhibit their own metabolism, or in situations where a patient must be maintained on a medication.

Nicotine is a drug which is chronically self-administered by smokers. Published studies of nicotine pharmacokinetics have required abstinence from tobacco prior to intravenous administration of unlabeled nicotine, in order for tobacco-derived nicotine concentration to decline to background levels.[2-4] This is not an ideal way to study nicotine metabolism, since abstinence from cigarette smoking may influence nicotine metabolism.[5] To study the quantitative metabolic disposition of nicotine during smoking, we synthesized a stable isotope-labeled analog with the same configuration as natural nicotine, (S)-nicotine-3',3'-d$_2$,[6] to administer intravenously to human smokers. This paper describes a selected ion monitoring method for simultaneous determination of nicotine, nicotine-3',3'-d$_2$, and the metabolites cotinine and cotinine-4',4'-d$_2$ in human plasma. Absence of an isotope effect in the clearance of nicotine-3',3'-d$_2$ in humans is also demonstrated, which validates the use of this analog for studies of nicotine pharmacokinetics.

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EXPERIMENTAL

Standards and reagents

Nicotine tartrate[7] and cotinine perchlorate[8] used as analytical standards were synthesized as previously described. Purity was verified by melting point, microanalysis for C, H and N, and analysis by gas chromatography/mass spectrometry (GC/MS).[8] (S)-Nicotine-3',3'-d$_2$, (S)-cotinine-4',4'-d$_2$, (±)-nicotine-3',3'-d$_2$-N'-methyl-d$_3$ and (±)-cotinine-3',3'-d$_2$-N'-methyl-d$_3$ were synthesized and purified by previously reported methods.[6] Toluene and 1-butanol were Fisher high-performance liquid chromatography (HPLC) grade; sulfuric acid was ACS reagent grade. Water used in preparation of reagent solutions was distilled from dilute chromic acid.

Instrumentation

GC/MS analyses were carried out using a Hewlett Packard 5890A gas chromatograph with a 7673 automatic liquid sampler, a split-splitless capillary inlet system, and a capillary direct interface to a quadrupole mass spectrometer, Hewlett Packard 5970B. Data were stored and processed using a Hewlett Packard 59970 MS Chem Station. GC analyses were performed using a Hewlett Packard 5880A instrument with a 7672 automatic sampler, a split-splitless capillary inlet system, nitrogen-phosphorus detector, and Level IV computing integrator.

Extraction procedure (Fig. 1)

To 1 ml aliquots of plasma samples, aqueous standards or spiked plasma controls in 13 × 100 mm glass culture

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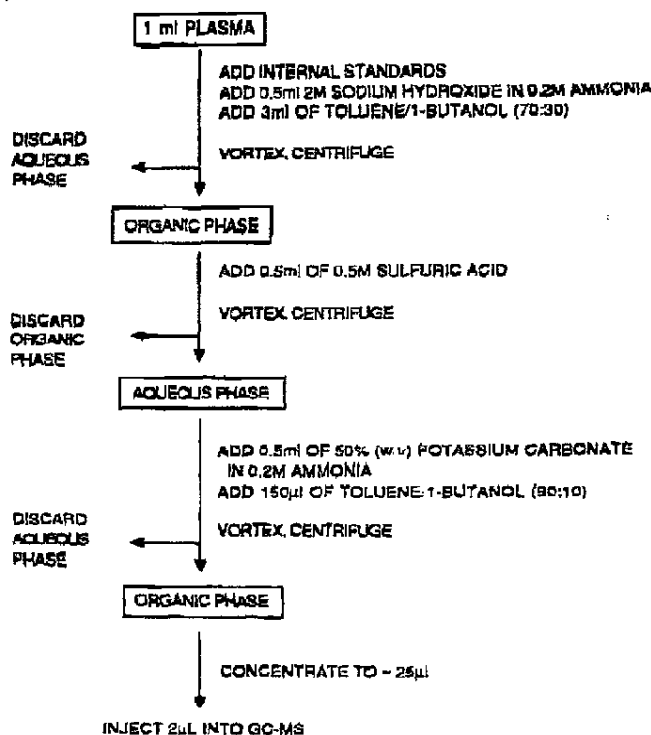

Figure 1. Extraction procedure.

tubes was added a mixture of the internal standards, 20 ng of (±)-nicotine-3',3'-d₂-N'-methyl-d₂ (nicotine-d₄) and 200 ng of (±)-cotinine-4',4'-d₂-N'-methyl-d₂ (cotinine-d₄) in 100 μl of 0.01 M HCl. The tubes were mixed 5 min on a vortex mixer, and then 0.5 ml of 2 M NaOH containing 0.2 M ammonia was added. A mixture of toluene and 1-butanol (70:30, 3 ml) was added, the tubes were capped and vortex-mixed for 5 min. The tubes were centrifuged to break emulsions, and then placed in a dry ice–acetone bath to freeze the aqueous layer. The organic layers were poured into culture tubes containing 0.5 ml of 0.5 M sulfuric acid. The tubes were vortex-mixed for 5 min, centrifuged, and placed in a dry ice–acetone bath to freeze the aqueous layers. Then the organic layers were poured off and discarded. Aqueous potassium carbonate (0.5 ml of 50% w/v containing 0.2 M ammonia) and 90:10 toluene–butanol (150 μl) were added, the tubes were vortex-mixed, centrifuged, and again placed in dry ice–acetone to freeze the aqueous layers. The organic layers were poured into 300 μl glass autosampler microvials and the extracts were then concentrated to about 25 μl by heating the microvials in a heating block at 85 °C. The vials were capped with aluminum foil and placed in the autosampler tray for GC/MS analysis.

GC/MS analysis

The injection (2 μl) was made in the splitless mode, using glass injection port liners containing a small plug of glass wool. The glass wool and injection port liner had been previously deactivated by soaking in 0.2% methanolic polyethylene glycol 4000 followed by drying in an oven for 0.5 h at ~80 °C. The injection port temperature was 250 °C, the carrier gas (helium) flow rate was 1 ml/min⁻¹, and septum purge on-time was 0.8 min. Separations were carried out on a Hewlett-Packard 12 m × 0.2 mm fused-silica capillary column coated with a 0.33 μm film of cross-linked 5% phenyl-methylsilicone, temperature programmed from 70 °C to 250 °C at 25 °C min⁻¹ following an initial hold for 1 min. The temperature of the transfer line to the mass spectrometer was calibrated with perfluoro-tributylamine (PFTBA) using the HP software 'Auto-tune' program. Then, ion current at m/z 69 of PFTBA was maximized by adjusting the ion focus setting. The electron multiplier was programmed to carry out analyses at 200 V above the Autotune value. Ionization was in the electron impact (EI) mode at 70 eV. Analyses of nicotine and nicotine-d₃ were carried out by monitoring the most abundant ions produced by EI ionization of the analytes and internal standard, m/z 84, 86 and 88, which result from loss of the pyridine ring. The molecular ions (m/z 176, 178, 180) were monitored for cotinine, cotinine-d₃ and the internal standard, cotinine-d₄. The ions were monitored at a mass peak width of 0.9 amu with a dwell time of 50 ms, in two groups: m/z 84, 86 and 88 from 4.0 to 4.5 min, and m/z 176, 178 and 180 from 6.1 to 6.7 min.

Quantification was achieved by integration of the ion chromatograms and constructing four-point standard

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curves of response (peak area ratio of analyte/internal standard) versus concentration, by linear regression. Standard curves were linear from 1 to 100 ng ml^{-1} for nicotine and nicotine-d$_2$, and from 10 to 1000 ng ml^{-1} for cotinine and cotinine-d$_3$, which spans the ranges of concentrations found in human plasma. Standard curves prepared from aqueous standards and spiked plasma standards were virtually identical. Equations for typical standard curves were: nicotine-d$_0$ in plasma, $y = 0.0965x + 0.0632$, $r^2 = 0.999999$; aqueous nicotine d$_0$, $y = 0.0937x + 0.0152$, $r^2 = 0.9997$; nicotine-d$_2$ in plasma, $y = 0.0951x + 0.0492$, $r^2 = 0.999999$; aqueous nicotine d$_2$, $y = 0.0921x + 0.0398$, $r^2 = 0.9998$; cotinine-d$_0$ in plasma, $y = 0.0066x + 0.000397$, $r^2 = 0.99998$; aqueous cotinine-d$_0$, $y = 0.0065x + 0.0065$, $r^2 = 0.9998$; cotinine-d$_3$ in plasma, $y = 0.0062x + 0.0519$, $r^2 = 0.99998$; aqueous cotinine-d$_3$, $y = 0.0062x + 0.0574$, $r^2 = 0.9998$. Due to the difficulty of obtaining plasma completely free from nicotine and cotinine, aqueous standards may be used instead of spiked plasma. Sample injections, data acquisition and analyte concentration determinations were carried out automatically using the HP Chem Station sequencing software and macro programs.

Precision and accuracy

Precision and accuracy were determined by analyzing plasma from non-smokers spiked with known concentrations of the four analytes (Table 1). In addition, concentrations of nicotine-d$_0$ and cotinine-d$_0$ determined by GC with nitrogen-phosphorus detection[7] were com-

Table 1. Intra-day assay precision and accuracy for nicotine, nicotine-d$_2$, cotinine and cotinine-d$_3$ in plasma

	Concentration (ng ml^{-1}) Actual	Measured[a]	Accuracy[a] (%)	CV[b] (%)
Nicotine	1	0.95	95	3.1
	2	2.02	101	3.4
	5	4.90	98	2.6
	20	20.1	101	1.3
	40	40.1	100	0.8
Nicotine-d$_2$	1	0.99	99	3.9
	2	2.05	103	1.9
	5	4.99	100	1.7
	20	20.2	101	0.8
	40	40.7	102	1.1
Cotinine	10	9.3	93	2.7
	20	19.1	96	2.2
	50	48.0	96	1.8
	200	197.1	99	0.8
	400	392.0	98	1.0
Cotinine-d$_3$	10	9.1	91	5.6
	20	19.1	96	2.8
	50	48.0	96	1.6
	200	196.7	98	0.9
	400	391.6	98	1.2

[a] Based on 6 replicate analyses.
[b] Coefficient of variation.

Table 2. Analysis of pooled smokers' plasma by GC and GC/MS

		Nicotine (ng ml^{-1})	Cotinine (ng ml^{-1})
Pool 1	GC/MS (mean of 4 analyses)	13.4	196
	GC (mean of 5 analyses)	12.2	222
	Mean, GC/MS and GC	12.8	209
	Percentage deviation from mean	4.7%	6.2%
Pool 2	GC/MS (mean of 4 analyses)	13.3	226
	GC (mean of 10 analyses)	14.0	196
	Mean, GC/MS and GC	13.7	211
	Percentage deviation from mean	2.6%	7.1%

pared with concentrations determined (Table 2) by the GC/MS method described in this paper.

Clinical studies

The elimination kinetics of labeled and natural nicotine were compared in five healthy male smokers, 29-61 years of age. These subjects smoked an average of 39 cigarettes per day (range 30-50). The subjects were hospitalized in the General Clinical Research Center at the San Francisco General Hospital Medical Center. After overnight abstinence from smoking, a 50:50 mixture of (S)-nicotine and (S)-nicotine-d$_2$ in a dose of 2 µg kg^{-1} min^{-1} (total nicotine base) was infused for 90 min. Plasma samples were collected during and for 6 h after the end of the infusion.

The data were analyzed using standard pharmacokinetic techniques. Elimination half-life and rate constant (k) were computed by linear regression of the log plasma nicotine concentration versus time curve in the terminal log-linear phase. The area under the plasma concentration-time curve (AUC) for nicotine was computed by the trapezoidal rule, with extrapolation of the terminal portion to infinity. Area contributed by the presence of natural nicotine before the infusion was computed as C_0/k, where C_0 was the preinfusion concentration, and was subtracted from the total AUC to get a net AUC, which was then used to compute clearance (CL). Clearance was computed as CL = DOSE/AUC. Steady-state volume of distribution was computed by a model-independent method.[9] Pharmacokinetic comparisons between natural and labeled nicotine were performed by paired t-tests.

RESULTS AND DISCUSSION

Methods have been reported previously for the determination of unlabeled nicotine and cotinine in biological fluids by selected ion monitoring GC/MS.[10-15] These have generally involved EI ionization, although for low concentrations of cotinine in tissue homogenates chemical ionization was reported to be advantageous.[12] Using a combination of capillary GC and high-resolution mass spectrometry, femtogram sensitivity for nicotine has been reported.[13]

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(S)-Nicotine

(S)-Cotinine

(S)-Nicotine - D$_2$

(S)-Cotinine - D$_2$
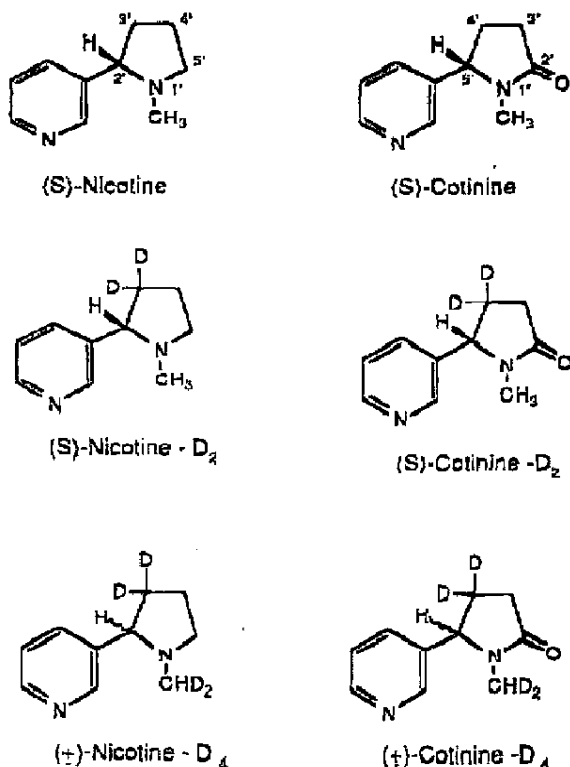
(±)-Nicotine - D$_4$

(±)-Cotinine - D$_4$

Figure 2. Structures of nicotine, cotinine and deuterium-labeled analogs.

The goal of the studies described in this paper was to develop a method for determination of nicotine, cotinine and deuterium-labeled analogs that could be applied to large numbers of samples generated in human pharmacokinetic studies. To study nicotine metabolism quantitatively concurrent with tobacco use, we synthesized (S)-nicotine-3',3'-d$_2$ for intravenous infusion in humans.[6] The 3'-position (Fig. 2) was chosen for incor-

poration of the label, since this position remains intact in the known primary metabolites of nicotine, and thus the elimination of nicotine-3',3'-d$_2$ would not be expected to be subject to a kinetic isotope effect.

The extraction scheme is shown in Fig. 1. Mixtures of toluene and 1-butanol were used as solvents, since they are effective for nicotine and cotinine extraction and remain liquid at —78 °C, allowing phase separations to be carried out by freezing the aqueous layers in a dry ice–acetone bath. To achieve the required sensitivity, it was necessary to concentrate the final extract to a volume of about 25 µl using a heating block. Chromatography was carried out using a 12 m × 0.2 mm i.d. 5% phenylmethylsilicone capillary column with splitless injection. Sharp peaks with good symmetry for analytes and internal standards were generally maintained for more than 100 consecutive injections. When peak symmetry began to degrade, the injection port liner was replaced with a clean glass liner containing a small plug of polyethylene glycol 4000 deactivated glass wool. This generally restored peak symmetry, but it was occasionally necessary to break off a 20–30 cm segment of column from the injection port end.

For determination of nicotine and nicotine-d$_2$, ions of m/z 84, 86 and the internal standard, m/z 88, were monitored (Fig. 3). These ions, which result from loss of the pyridine ring, are the most abundant ions produced by EI ionization of nicotine and the deuterium-labeled analogs.[6] No significant interfering peaks were observed in the ion chromatograms derived from extracts of non-smokers' plasma, allowing the determination of nicotine and nicotine-d$_2$ concentrations as low as 1 ng ml^{-1}.

Since other investigators[15] reported less interference from endogenous substances in plasma if the molecular ion (m/z 176) of cotinine was monitored, we analyzed 26 samples by monitoring the molecular ions (m/z 176 and 178) of cotinine and cotinine-d$_2$, as well as the most abundant ions, m/z 98 and 100. For these samples, which were obtained from smokers following an intravenous infusion of nicotine-d$_2$, cotinine concentrations determined by monitoring m/z 98 were highly correlated with concentrations determined from m/z 176

NICOTINE
M/Z 84

COTININE
M/Z 176

NICOTINE-D2
M/Z 86

COTININE-D2
M/Z 178

NICOTINE-D4 ISTD
M/Z 88

COTININE-D4 ISTD
M/Z 180

4.0   4.5   6.2   6.9
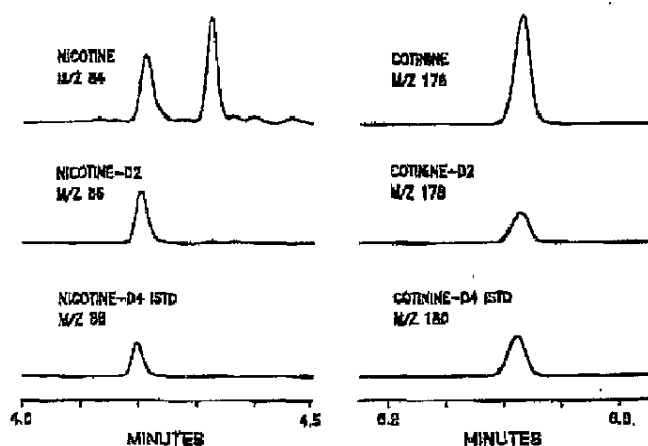MINUTES   MINUTES

Figure 3. Ion chromatograms of an extract of plasma from a smoker following intravenous infusion of (S)-nicotine-3',3'-d$_2$, containing 29 ng ml^{-1} nicotine, 16 ng ml^{-1} nicotine-d$_2$, 400 ng ml^{-1} cotinine and 82 ng ml^{-1} cotinine-d$_2$.

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Table 3. Relative abundances[a] of m/z 84, 86 and 88 ions produced by electron ionization of nicotine isotopomers			
	84	86	88
Nicotine-d₀	100	0.23	0.16
Nicotine-d₂	1.82	100	0.26
Nicotine-d₄	0.71	2.81	100

[a] Determined by selected ion monitoring and integration of the ion chromatograms.

Table 4. Relative abundances[a] of m/z 176, 178 and 180 ions produced by electron ionization of cotinine isotopomers			
	176	178	180
Cotinine-d₀	100	0.91[b]	ND[c]
Cotinine-d₂	2.84	100	1.07
Cotinine-d₄	0.22	6.73	100

[a] Determined by selected ion monitoring and integration of the ion chromatograms.
[b] Theoretical value = 0.84.[17]
[c] None detected.

($r^2 = 0.998$), and were not significantly different. Likewise, concentrations of cotinine-d₂ determined by monitoring m/z 178 and m/z 100 were highly correlated ($r^2 = 0.997$), although there was a tendency for concentrations based on the molecular ion to be slightly lower. It was noted that the m/z 176 and 178 ion chromatograms derived from non-smokers' plasma and aqueous blank samples were cleaner, which indicates that the molecular ions are more suitable for determination of low cotinine concentrations.

Concentrations were determined using the peak area ratio of the analyte to its respective tetradeuterated internal standard, and constructing standard curves by linear regression. This was possible because of the low degree of ion overlap (Tables 3 and 4) and because the concentrations of nicotine and nicotine-d₂ were similar in our study. However, in cases in which concentrations of nicotine and nicotine-d₂ (or cotinine and cotinine-d₂) differ by several fold, it would be necessary to calculate concentrations using equations that take into account ion overlap,[16] or to correct concentrations of the minor component for contributions due to ion overlap with the major component.

Accuracy and precision of the method were evaluated by carrying out replicate analyses of spiked non-smokers' plasma. Coefficients of variation for nicotine and nicotine-d₂ were less than 4% for concentrations ranging from 1 to 40 ng ml⁻¹, which spans the range of concentrations generally found in smoker's plasma. For cotinine and cotinine-d₂, the coefficients of variation were less than 6% from 10 to 400 ng ml⁻¹, which like-

wise covers the range typically found in smokers' plasma. Accuracy ranged from 95 to 102% for nicotine and nicotine-d₂ and from 91 to 99% for cotinine and cotinine-d₂ (Table 1). In addition, concentrations of nicotine and cotinine in pooled smokers' plasma determined by GC with nitrogen-phosphorus detection correlated well with concentrations determined by GC/MS (Table 2).

To determine the validity of using (S)-nicotine-3′,3′-d₂ in studies of nicotine metabolism in humans, we compared the elimination kinetics of labeled and natural nicotine in five smokers. This was carried out by administering an intravenous infusion of a 50:50 mixture of nicotine and nicotine-d₂ in a dose which produces plasma nicotine levels similar to those of heavy cigarette smokers. Mean plasma concentrations of nicotine and nicotine-d₂ during and after the infusion are shown in Fig. 4. Total clearance was virtually identical for labeled and natural nicotine (Table 5). The terminal half-life ($t_{1/2}\beta$) was slightly longer for nicotine-d₀, possibly resulting from slow release of tobacco-derived nicotine from deep tissue stores. The volume of distribution of nicotine-d₂ was similar to the volume of distribution of natural nicotine.

In summary, a selected ion monitoring method is described for simultaneous determination of nicotine, cotinine and deuterium-labeled analogs in human plasma. Sample injection, data acquisition and calculation of analyte concentration have been automated, which has facilitated the use of the method in large-scale clinical studies. Demonstration that the clearance

Table 5. Disposition kinetics of natural nicotine and (S)-nicotine-3′,3′-d₂ in five smokers[a]

Subject	Total clearance (ml min⁻¹)		Half-life, min ($t_{1/2}\beta$)		Volume of distribution l	
	Nicotine	Nicotine-d₂	Nicotine	Nicotine-d₂	Nicotine	Nicotine-d₂
1	1200	1210	216	178	319	284
2	1420	1500	167	122	317	265
3	1110	1210	196	165	303	274
4	1560	1610	143	139	304	298
5	1730	1570	110	144	273	264
Mean	1404	1420	166	149	303	277
S.D.	254	186	39	22	18	18

[a] Difference in pharmacokinetic parameters comparing natural and labeled nicotine was not significant by paired t-test.

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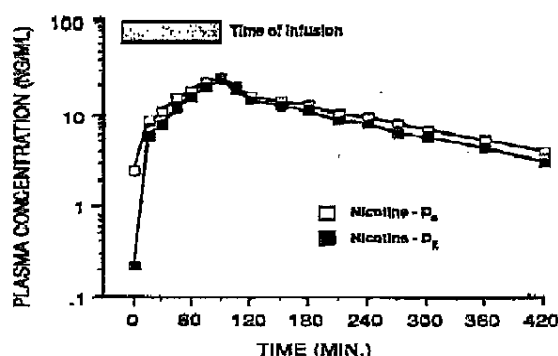252   P. JACOB, III, L. YU, M. WILSON AND N. L. BENOWITZ

Figure 4. Mean plasma concentrations of nicotine and nicotine-d_2 in five human subjects during and after intravenous infusion of a 50:50 mixture of nicotine and nicotine-d_2.

of (S)-nicotine-3',3'-d_2 is virtually identical to the clearance of natural nicotine validates the use of this analog for quantitative studies of nicotine metabolism.

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